

CHARACTERIZATION OF PROTEIN-PROTEIN INTERACTION WITHIN A  
POLYAMINE RESPONSIVE SIGNALING SYSTEM IN *VIBRIO CHOLERAE*

A Thesis

by

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## ABSTRACT

### CHARACTERIZATION OF PROTEIN-PROTEIN INTERACTION WITHIN A POLYAMINE RESPONSIVE SIGNALING SYSTEM IN *VIBRIO CHOLERAE* (August 2012)

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The Gram-negative bacterium *Vibrio cholerae* is a natural inhabitant of aquatic environments and the causative agent of the severe diarrheal disease cholera. This bacterium is able to form biofilms on both abiotic surfaces and on the biotic surfaces of chitinous zooplankton. Biofilm formation is vital to the survival of *V. cholerae* both within aquatic reservoirs and during transmission to the human host. The bacterial second messenger cyclic-di-GMP is an important signaling system that is known to regulate biofilm formation in response to environmental signals. Previous research investigating a putative c-di-GMP-signaling pathway involving the periplasmic protein NspS and the GGDEF-EAL protein MbaA, revealed that the polyamines spermidine and norspermidine likely act as specific extracellular environmental signals to modulate NspS-MbaA interaction and affect biofilm formation. These proteins have opposite effects on biofilm formation *in vitro* and are encoded by adjacent genes with overlapping reading frames as part of an operon structure.

The objective of this study has been to provide evidence of direct protein-protein interaction between NspS and MbaA, and to further elucidate the molecular details of the

putative norspermidine/spermidine-MbaA/NspS signaling system in *V. cholerae*. Our results further characterize the role periplasmic polyamine-binding proteins play in cyclic-di-GMP-mediated signal transduction within the first polyamine-responsive signaling system identified in bacteria.

## DEDICATION

This work is dedicated to my parents, Sandy Lee Pendergraft and Lee Anne Stanley Pendergraft, whose love and support have never waived.

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## TABLE OF CONTENTS

Abstract.....	iv
Acknowledgments.....	vii
List of Tables.....	ix
List of Figures.....	x
Introduction.....	1
Materials and Methods.....	6
Results.....	18
Discussion.....	36
References.....	43
Biographical Sketch.....	46



## LIST OF TABLES

Table 1. Strains and Plasmids.....	17
Table 2. Primers.....	17

## LIST OF FIGURES

Fig. 1. Genomic architecture and predicted cellular locations of NspS and MbaA.....	4
Fig. 2A. Genomic architecture and predicted cellular locations of NspS and MbaA.....	19
Fig. 2B. Flowchart of cloning of a fragment encoding C-terminal MbaA protein.....	20
Fig. 2C. Confirmation of the <i>mbaA</i> insert in pET-28b(+):MbaA6His construct.....	20
Fig. 3. Expression and localization of recombinant MbaA C-terminal protein.....	22
Fig. 4. Affinity purification of the polyclonal MbaA antibody.....	23
Fig. 5. Detection of MbaA in whole cell extracts .....	24
Fig. 6. Silver stain of the immunoprecipitation complex using V5 agarose.....	26
Fig. 7. Effect of BS <sup>3</sup> crosslinking on <i>V. cholerae</i> and <i>V. cholerae</i> w/ $\Delta$ <i>mbaA</i> .....	27
Fig. 8. Effect of BS <sup>3</sup> crosslinking on NspS dimerization and complex formation.....	27
Fig. 9. Coimmunoprecipitation of NspS/MbaA using anti-MbaA antibody.....	28
Fig. 10A. Polyamine effect on MbaA/NspS complex in <i>V. cholerae</i> .....	30
Fig. 10B. Polyamine effect on MbaA/NspS complex in <i>V. cholerae</i> w $\Delta$ <i>mbaA</i> .....	30
Fig. 11. Flowchart of cloning and ligation of the <i>nspS</i> gene using pET-26b(+). ....	31
Fig. 12. Amplification of <i>nspS</i> insert using Phusion® Polymerase.....	31
Fig. 13. Confirmation of the <i>nspS</i> insert in pET-26b(+):NspS6His construct.....	32
Fig. 14. Expression and localization of recombinant NspS protein.....	33
Fig. 15. General purification scheme using HisPur cobalt resin to purify NspS.....	34
Fig. 16. Purification of periplasmic NspS protein using HisPur cobalt resin.....	35

## INTRODUCTION

The Gram-negative proteobacterium *Vibrio cholerae* exists as both a free-living environmental fauna organism and a human intestinal pathogen. This bacterium is the causative agent of the severe diarrheal disease cholera, and a natural inhabitant of oceans, lakes, rivers, and estuaries where it is believed to exist predominantly as matrix-enclosed, surface-associated communities known as biofilms (Colwell and Huq, 1994). Biofilms are composed of bacteria embedded in a matrix of proteins, DNA, and an exopolysaccharide made up mainly of galactose and glucose (Yildiz & Schoolnik, 1999). Biofilms confer a marked survival advantage in hostile environments by providing protection against damaging sources such as antibiotics, pH changes including those within the human digestive tract, and host defense mechanisms (Kamruzzaman *et al.*, 2010; Hood & Winter, 1997; Mah & O'Toole, 2001; Hung *et al.*, 2006). This biofilm state has been correlated with increased persistence and survival of *V. cholerae* within its aquatic environment (Huq *et al.*, 2008), yet biofilms are also important for the survival and propagation of this bacterium within the human host (Yildiz & Visick, 2009). Recent evidence indicates that biofilm-specific protein antibodies are present in people that have been infected with cholera (Hang *et al.*, 2003), and that bile induces the expression of several biofilm-specific genes (Hung *et al.*, 2006).

Biofilm formation appears to be necessary for effective cholera disease transmission, and it is believed that human ingestion of *V. cholerae* occurs primarily in

biofilm form (Faruque *et al.*, 2006). The biofilm matrix protects the bacteria during transit through the stomach to the small intestine. However, *V. cholerae* bacteria must exit the biofilm and regain motility in order to colonize the small intestine of its host (Hughes *et al.*, 1982; Schild *et al.*, 2007). The bacterium, upon colonization of the small intestine, releases vast amounts of cholera toxin, an enterotoxin that elicits the characteristic watery diarrhea associated with the disease (Satchell, 2003).

The regulation of the transition between biofilm formation, virulence, and motility is highly regulated by numerous proteins and cues in the cell that respond to environmental signals (Hammer & Bassler, 2003; Nikolaev & Plakunov, 2007). Some of the proteins involved in this process have been partially characterized (Bomchil *et al.*, 2003; Karatan *et al.*, 2005); however, how they specifically interact with each other to regulate *V. cholerae* biofilm formation is yet to be elucidated, and many of the specific details regarding these signaling mechanisms remain unknown.

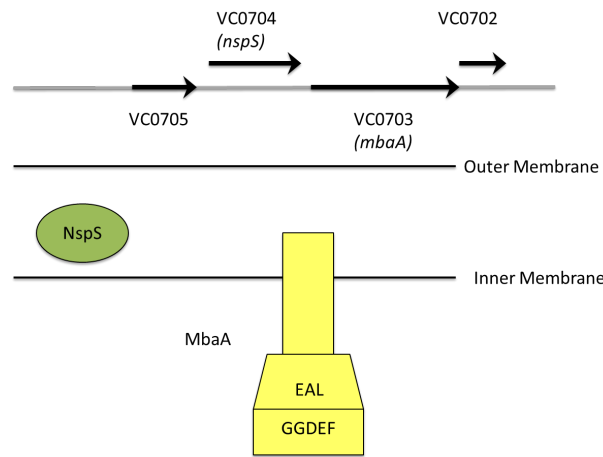
However, it is known that many of the environmental signals and cellular networks that regulate biofilm formation do so by affecting cellular levels of the bacterial second-messenger cyclic (5' to 3')-diguanosine monophosphate (c-di-GMP). Cyclic di-GMP is ubiquitous in bacteria and implicated in the regulation of numerous complex physiological processes (Romling & Amikam, 2006). This molecule has been found to be the main controller of the switch between biofilm and planktonic lifestyles in Gram-negative bacteria (Simm *et al.*, 2004; Tischler & Camilli, 2004). Cyclic di-GMP is synthesized by diguanylate cyclase enzymes containing conserved GGDEF enzymatic domains. These enzymes bind two molecules of GTP and cyclize them into cyclic di-GMP (Chan *et al.*, 2004; Ryjenkov *et al.*, 2005). The break down of cyclic di-GMP is

catalyzed by phosphodiesterase enzymes containing conserved EAL or HD-GYP enzymatic domains. These protein domains function to break down cyclic di-GMP into pGpG via phosphodiesterase activity (Schmidt *et al.*, 2005; Ryan *et al.*, 2006). It has been shown in previous studies that low levels of cyclic di-GMP triggers an increase in motility, while high levels cause an increase in biofilm formation in *V. cholerae* (Jenal & Malone, 2006; Romling & Amikam, 2006). Evidence linking biofilm formation, cyclic di-GMP, and GGDEF/EAL/HD-GYP domains has revealed that when a specific GGDEF-domain protein in *V. cholerae* is overexpressed it directly upregulates the *V. cholerae* *vpsA-Q* genes involved in exopolysaccharide matrix production, and that this upregulation is directly associated with increased cyclic-di-GMP levels (Chang *et al.*, 2001; Hammer & Bassler, 2003; Tischler & Camilli, 2004).

Recent characterization has revealed that a putative signaling complex in *V. cholerae* O139 composed of the periplasmic protein NspS and the putative integral membrane protein MbaA likely functions to regulate biofilm formation by affecting cellular c-di-GMP levels in response to environmental norspermidine and spermidine levels (Fig. 1) (Karatan *et al.*, 2005; McGinnis *et al.*, 2009). Norspermidine and spermidine are part of a class of organic polycations called polyamines. Polyamines are small hydrocarbon chains containing two or more primary amino groups. These molecules are positively charged at physiological pH, and are essential for normal growth in both prokaryotes and eukaryotes.

It is thought that norspermidine binds the periplasmic protein NspS, and that NspS interacts with the transmembrane protein MbaA and likely functions in the cyclic-di-GMP biofilm regulatory pathway. This was concluded because deletion of the *mbaA*

gene leads to an increase in both biofilm and activity of genes encoding *Vibrio* polysaccharide synthesis and export proteins (Bomchil *et al.*, 2003; Karatan & Watnick, 2009; Lim *et al.*, 2007). The NspS protein is proposed to function in this pathway since its ability to sense the polyamine norspermidine is associated with biofilm regulation, and its deletion leads to a drastic decrease in biofilm in *V. cholerae* (Karatan *et al.*, 2005). The specific putative interaction between these two proteins is hypothesized to occur in the periplasmic space since NspS is a periplasmic protein and MbaA has a large periplasmic domain. This idea is illustrated in figure 1.



**Figure 1. Genomic architecture and predicted cellular locations of NspS and MbaA.**

Research into polyamine regulation of *V. cholerae* biofilms and specifically targeting polyamines with certain pharmaceutical drugs has been implicated as possible novel treatment options for cholera disease (Van Dellen & Watnick, 2006).

The objective of this study has been to provide evidence of definite interaction between constituents of a putative polyamine responsive signaling system in *V. cholerae* O139 composed of the recognized periplasmic protein NspS and the putative integral membrane protein MbaA. These proteins are encoded by adjacent genes and cotranscribed as part of an operon structure (Porter, 2009). A revealing aspect of this

system is the presence of both GGDEF and EAL domain motifs on the C-terminal portion of the MbaA protein. Because of this, we believe that MbaA likely controls dedicated c-di-GMP pools within the cell. Since MbaA has been shown to reduce biofilm, it is likely functioning as a phosphodiesterase.

In this work we have further elucidated the molecular details of the putative norspermidine/spermidine-MbaA/NspS signaling system. We provide evidence of a direct NspS-MbaA interaction within this system. This research further characterizes the role periplasmic polyamine-binding proteins play in cyclic-di-GMP-mediated signal transduction within the first polyamine-responsive signaling system identified in bacteria.

## MATERIALS AND METHODS

### ***Bacterial strains, plasmids, media, and reagents***

All bacterial strains and plasmids are listed in Table 1. All experiments were conducted with Luria-Bertani (LB) broth or agar with appropriate antibiotic unless otherwise indicated. *V. cholerae* serotype O139 strain MO10 was used for all experiments. Restriction endonucleases, T4 DNA ligase, and Phusion DNA polymerase were from New England Biolabs (Beverly, MA). Polyamines were purchased from Sigma (St. Louis, MO). The V5 antibody was purchased from Serotec (Raleigh, NC). Primer synthesis was done by MWG Biotech (High Point, NC). DNA sequencing was performed by Life Sciences core facility at Cornell University (Ithaca, NY). Primers used for amplification, mutagenesis, and cloning are listed in Table 2.

### ***Cloning, expression, and purification of MbaA for polyclonal antibody production***

Construct Design—The portion of the *mbaA* gene encoding the C-terminal cytoplasmic domain corresponding to amino acids 260-760 was PCR-amplified using primers PA195 and PA196 from chromosomal *V. cholerae* DNA with Phusion® High-Fidelity DNA Polymerase. These primers were designed using Vector NTI Suite (Life Technologies, Grand Island, NY) to add flanking *NdeI* and *XhoI* restriction enzyme recognition sites to the DNA construct. The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Seven µl of the purified PCR product was



digested in a total reaction volume of 20  $\mu$ l containing 2  $\mu$ l NEB buffer 4, 0.5  $\mu$ l *Nde*I, 0.5  $\mu$ l *Xho*I, 0.5  $\mu$ l Rnase and 9.5  $\mu$ l H<sub>2</sub>O for 3 hours at 37°C and run on a 1% agarose gel. The resulting ~1500bp *mbaA* insert was gel purified using Wizard<sup>®</sup> SV Gel and PCR Clean-Up System (Promega) and ligated into *Nde*I and *Xho*I digested and gel purified pET-28b(+)vector (Novagen, Madison, WI) encoding an additional C-terminal polyhistidine tag for affinity purification. The ligation of pET-28b(+) and *mbaA* was performed by the addition of 5 $\mu$ l 10X T4 DNA ligase buffer, 4 $\mu$ l digested pET28(b) vector, 10 $\mu$ l digested *mbaA* insert, and 1 $\mu$ l T4 ligase at a total volume of 20  $\mu$ l with incubation at 16°C, overnight. The ligation was dialyzed against water using a Millipore dialysis disk for 30 minutes. The plasmid was electroporated into DH5 $\alpha$  cells using a BIO-RAD MicroPulser (Hercules, CA) at 1.8 kV. Cells were incubated in 2mL SOC media (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl<sub>2</sub>, 10mM MgSO<sub>4</sub>, 20mM D-Glucose) at 37° C for 3 hours at 180 rpm, and then plated on LB-Kanamycin (50  $\mu$ g/mL) plates. Colony PCR was used for identification of positive colonies. A single colony was resuspended in 100 $\mu$ L deionized water in a 0.2ml PCR tube (ISC BioExpress, Kaysville, U.T.). Bacterial cells were lysed by denaturation at 95°C for 5 minutes in an Eppendorf Mastercycler (Eppendorf, Hauppauge, NY). Cellular debris was removed by centrifugation at 10,000 x g for 5 minutes. Two  $\mu$ L of the supernatant containing the template DNA, 5  $\mu$ L 5X *OneTaq* Standard Reaction Buffer 0.75  $\mu$ L 10mM dNTPs, 0.2  $\mu$ M of primers PA195 and PA196, and 0.125  $\mu$ L *OneTaq* DNA polymerase was added at a total reaction volume of 25  $\mu$ L. PCR settings were as follows: initial denaturation at 95° C for 5 minutes, 95° C for 30 seconds (30 cycles), primer annealing at 60° C for 30 seconds, an extension step at 72° C for 60 seconds, and

a final extension at 72° C for 5 minutes. Colonies positive for the insert were grown as 2mL overnight cultures with 50 µg/mL kanamycin. Plasmids were purified using the Promega Wizard Plus SV Minipreps DNA Purification System. The sequence of the final construct was verified by DNA sequence analysis and the plasmid was transformed into chemically competent BL21(DE3) cells. Transformation was completed by the addition of 1µl of plasmid to 50µl BL21 cells, and subjected to heat shock of 42°C for 90 seconds. Cells were immediately incubated on ice for 3 minutes, then recovered for 3 hours with 200 rpm shaking at 37°C in 2mL SOC media and plated on LB agar plates containing 50 µg/ml kanamycin. The resulting strain, BL21(DE3) with the pET28(b)::MbaA6His plasmid construct, was then stored at -80 °C for downstream applications.

Protein Expression and Purification— To overexpress the C-terminus portion of MbaA, *E.coli* BL21(DE3) cells containing the pET28(b)::MbaA6His plasmid construct were grown at 37 °C in LB medium and induced at an OD<sub>595</sub> of 0.6 with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) for 16 h at 20°C. An initial pilot experiment was completed by inoculating a 2mL overnight culture of BL21(DE3) pET28(b)::MbaA6His into 50mL LB at a 1:25 dilution. This culture was grown and induced as described above and revealed that the protein was mostly expressed as insoluble inclusion bodies. The experiment was then scaled up to 1 liter. One liter of LB was inoculated with a 20mL overnight culture of BL21(DE3) pET28(b)::MbaA6His at a 1:50 dilution, grown and induced as described previously. The Novagen pET system manual was followed for the purification of inclusion body proteins. Briefly, following induction, the cells were harvested at 10,000 x g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in BugBuster Master Mix (Novagen) at

5mL reagent per gram of wet cell paste. The cell suspension was incubated on a shaking platform at a slow setting for 20 minutes at room temperature. The suspension was centrifuged at 16,000 x g for 20 minutes at 4 °C. The resulting insoluble cell pellet was resuspended in the same volume of BugBuster Master Mix used to resuspend the original cell pellet. Following complete resuspension of the cell pellet, 6 volumes of BugBuster Master Mix diluted 1:10 in deionized water was added and vortexed for 1 minute. The suspension was centrifuged at 5000 x g for 15 min at 4 °C to collect the inclusion bodies. The supernatant was removed and the inclusion bodies were resuspended in half of the original culture volume of 1:10 diluted BugBuster Master Mix, vortexed for 1 minute and centrifuged at 5000 x g for 15 min at 4 °C. This step was repeated 3 more times with the final centrifugation being at 16,000 x g for 15 min at 4 °C. The supernatant was discarded and the inclusion bodies were resuspended in 1% SDS with heating and sonication. The inclusion bodies were analyzed by SDS-PAGE for purity and the MbaA C-terminal protein was cut from the gel and used directly for polyclonal antibody production by Lampire Inc. (Pipersville, PA). A total of 2mg of purified inclusion body protein was used for this purpose as determined by Bradford assay.

### ***Purification of polyclonal antibodies against MbaA C-terminal protein***

Affinity purification of total rabbit serum was completed using a Thermo Scientific Nab Spin Kit following manufacturer's instructions. Specifically, the serum sample was prepared by diluting 1.5 mL sera to 2 mL in binding buffer (100 mM phosphate, 150 mM sodium chloride; pH 7.2). A one mL immobilized protein A/G resin spin column was placed in a 15 mL collection tube and centrifuged for 1 minute at 1000

x g to remove storage solution. The spin column was then equilibrated by adding 2 mL binding buffer and centrifugation at 1000 x g for 1 minute. The bottom of the spin column was capped and the sample was loaded onto the column and incubated at room temperature with end-over-end mixing for 10 minutes. The column was placed into a new collection tube and centrifuged for 1 minute at 1000 x g. Flow through was saved for downstream analysis. Spin column was transferred to another collection tube and washed by the addition of 2 mL binding buffer and 1 minute centrifugations at 1000 x g for a total of 3 washes. One hundred microliters of Neutralization buffer (1 M Tris, pH 8.5) was added to 3 collection tubes and the spin column was placed into the first of these tubes. One milliliter of IgG Elution Buffer pH 2.8 was added to the spin column and centrifuged for 1 minute at 1000 x g. Three elution fractions were obtained from this process by moving the spin column to the 2<sup>nd</sup> and 3<sup>rd</sup> collection tubes containing Neutralization buffer and centrifugation. The total IgG concentration of each elution fraction was determined using the IgG reference option on a NanoDrop 1000 spectrophotometer.

### ***Cloning, expression, and purification of NspS***

Construct Design—The *nspS* gene was amplified from chromosomal *V. cholerae* DNA with Phusion® High-Fidelity DNA Polymerase without its native signal sequence as predicted by SignalP 4.0 using primers PA184 and PA197. This construct corresponded to amino acids 34-359 with primers adding flanking *NcoI* and *XhoI* restriction enzyme recognition sites to the DNA construct. The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and then was digested with

*NcoI* and *XhoI*. The *nspS* insert was digested by the addition of 7µl DNA, 2µl NEB buffer 3, 0.5 µl *NcoI*, 0.5µl *XhoI*, 0.5µl Rnase and 9.5 µl H<sub>2</sub>O for 3 hours at 37°C. The resulting ~1091 bp *nspS* insert was gel purified using Wizard<sup>®</sup> SV Gel and PCR Clean-Up System (Promega) and ligated into the *NcoI* and *XhoI* digested and gel-purified pET26(b) vector (Novagen) encoding an N-terminal *pelB* signal sequence for periplasmic localization of the recombinant protein and an additional C-terminal polyhistidine tag for affinity purification. The ligation of pET26(b) and *nspS* was performed by the addition of 5µl 10X T4 DNA ligase buffer, 4µl digested pET26(b) vector, 10µl digested *nspS* insert, and 1µl T4 ligase with incubation at 16°C, overnight. The ligation was dialyzed against water using a Millipore dialysis disk for 30 minutes, and then electroporated into DH5α cells using a BIO-RAD MicroPulser (Hercules, CA) at 1.8 kV. Cells were incubated in 2mL SOC media at 37° C for 3 hours at 180 rpm, and then plated on LB-Kanamycin (50 ug/mL). Colony PCR was used for identification of positive colonies as described above. PCR setting were initial denaturation at 95° C for 5 minutes, 95° C for 30 seconds (30 cycles), primer annealing at 60° C for 30 seconds, an extension step at 72° C for 60 seconds, and a final extension at 72° C for 5 minutes. Colonies positive for the insert were grown as 2mL overnight cultures with 50 ug/mL kanamycin. Plasmids were purified using the Promega Wizard Plus SV Minipreps DNA Purification System. Plasmids were verified by restriction digest to confirm the presence of the *nspS* insert. DNA sequencing was then used to confirm that the entire insert sequence was correct with no mutations. The plasmids were then transformed into chemically competent BL21(DE3) cells as described above.

Protein Expression and Purification—The NspS6His protein was overexpressed in *E.coli* BL21(DE3). An initial pilot experiment was completed by inoculating a 2mL overnight culture of BL21(DE3) with pET-26b(+):NspS6His into 50mL LB at a 1:25 dilution. This culture was grown at 37 °C in LB medium and induced at an OD<sub>595</sub> of 0.6 with 1 mM IPTG for 4 h at 37°C and revealed that while the protein was mostly expressed as insoluble inclusion bodies, a significant amount was expressing in the periplasmic space. In order to select for the greatest expression of soluble periplasmic proteins various induction temperatures and IPTG concentrations were tested. Final IPTG concentrations of 0.2 mM, 0.5 mM, 0.8 mM, 1mM, and 1.2 mM and induction temperatures of 16°C, 20°C, 27°C, 30°C, and 37°C were tested for this purpose and it was determined that 1mM IPTG at 20°C was optimum for soluble periplasmic protein expression. The experiment was then scaled up to 1L. One liter of LB was inoculated with a 20mL overnight culture of BL21(DE3) with pET-26b(+):NspS6His at a 1:50 dilution, grown at 37 °C in LB medium and induced at an OD<sub>595</sub> of 0.6 with 1 mM IPTG for 16 h at 20°C. The Novagen pET system manual was followed for the extraction of periplasmic proteins. Following induction cells were harvested at 4000 x g for 20 minutes. The supernatant was discarded and the cell pellet was resuspended in 30 mM Tris-HCl, 20% sucrose, pH 8.0 at 80mL per gram wet weight. Cells were kept on ice and 0.4 ml of 500mM Ethylenediaminetetraacetic acid (EDTA) was added dropwise to a final concentration of 1mM. Cells were incubated on ice for 10 minutes with gentle agitation. This suspension was centrifuged at 8000 x g for 20 minutes at 4 °C. The supernatant was discarded and the cell pellet was resuspended in 200 mL of ice-cold 5mM MgSO<sub>4</sub> and shaken by rotary shaker for 10 minutes. This suspension was centrifuged at 8000 x g for

20 minutes at 4 °C and the supernatant was recovered as the osmotic shock fluid containing periplasmic proteins. The periplasmic fraction was concentrated to 10 mL using a Millipore Amicon Ultra-15 15mL spin concentrator (Millipore, Billerica, MA) (molecular mass cut-off, 3 kD) and dialyzed extensively against a buffer containing 50mM sodium phosphate, 300mM sodium chloride, 30mM imidazole at pH 7.4.

Recombinant NspS protein was purified from the dialyzed osmotic shock fluid using HisPur Cobalt Resin (Thermo Fisher Scientific, Hudson, NH) using a gravity-flow column. Briefly, a gravity-flow column was packed with 250 µl of HisPur cobalt resin and equilibrated with two resin-bed volumes of equilibration/wash buffer (50mM sodium phosphate, 300mM sodium chloride, 30mM imidazole; pH 7.4) at a flow rate of 0.5mL/minute. Protein sample was prepared by mixing with an equal volume of equilibration/wash buffer and loaded onto the column. The flowthrough was re-loaded to maximize binding. Resin was washed with two resin-bed volumes of equilibration/wash buffer until protein concentration of wash approached baseline (as determined by Bradford protein assay). Histadine-tagged NspS protein was eluted 3 times using one resin-bed volume of elution buffer (50mM sodium phosphate, 300mM sodium chloride, 150mM imidazole; pH 7.4). Protein purity was judged by SDS- PAGE and concentration measured by Bradford assay.

#### ***In vitro crosslinking by bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>)***

Bacterial strains were grown as 20mL overnight cultures with appropriate antibiotic at 37°C with shaking at 200 rpm. Cultures were harvested by centrifugation at 10,000 x g for 3 minutes. The resulting supernatant was discarded and the cell pellet was

washed three times with ice-cold PBS (20mM sodium phosphate, 0.15M NaCl, pH 8.0) to remove amine-containing culture media from the cells. Following the final wash, the pellet was resuspended in 500  $\mu$ l of PBS (pH 8.0). Bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>) crosslinker was prepared immediately before use and added to each cell suspension at a final concentration of 1mM. Reactions were incubated for 40 minutes at room temperature using a rotary shaker. Reactions were quenched upon addition of how many 15  $\mu$ l of 1M Tris, pH 7.5 at a final concentration of 20 mM. The quenching reaction was incubated for an additional 15 minutes before being sonicated with a 40% duty cycle for three 10-second pulses with at least 15 seconds between sonications using a Heat Systems Ultrasonics Sonicator Model W 380 (Farmingdale, NY). Samples were incubated on ice between pulses. The suspension was then centrifuged for 2 minutes at 16,000 x g and the resulting supernatant stored for downstream applications.

### **Co-immunoprecipitation**

*V5 Co-Immunoprecipitation*— 20 $\mu$ l of agarose-immobilized goat anti-V5 antibody (Bethyl Labs, Montgomery, TX) was incubated with 200  $\mu$ l BS<sup>3</sup> crosslinked whole cell protein extracts containing 120  $\mu$ g of total protein overnight at 4 °C with rotating. Each suspension was centrifuged for 1 min at 3000 x g. Supernatant was removed and saved as the unbound fraction. The V5-agarose was washed 3 times with a buffer containing 1% Triton-X-100 and 50mM Tris pH 8.0. Following the final wash, 20 $\mu$ l of 2X SDS loading buffer (0.5M Tris-HCl pH6.8, glycerol, 10% (w/v) Sodium Dodecyl Sulfate (SDS) (ISC BioExpress), 0.5% (w/v) bromophenol blue (International Biotechnologies Inc., New Haven, C. T.), 1.4% (v/v)  $\beta$ -Mercaptoethanol (Fisher



Scientific) was added and boiled for 10 minutes to denature the proteins. Samples were separated by SDS-PAGE analyzed via Western blotting as described below.

*MbaA Co-Immunoprecipitation*— 25µl of protein A/G agarose was incubated with 50µl affinity purified MbaA antibody overnight at 4 °C with rotating. The suspension was washed 3 times with 500µl 1X PBS to remove unbound antibody. 20µl of the protein A/G anti-MbaA antibody slurry was incubated with 200µl BS<sup>3</sup> crosslinked whole cell protein extracts overnight at 4 °C on a rotary shaker. Each suspension was centrifuged for 1 min at 3000 x g. Supernatant was removed and saved as the unbound fraction. The V5 agarose was washed 3 times with 500 microliters of a buffer containing 1% TritonX-100, 50mM Tris pH 8.0. Following the final wash 20µl of 2X SDS loading buffer was added and the sample was boiled for 10 minutes to denature the proteins. Samples were analyzed via Western blotting as described above.

### ***Western Blotting***

Samples were mixed 1:1 with 2X SDS loading buffer, boiled in water for 10 minutes, and run on an SDS-polyacrylamide gel with a 4-15% gradient for 40 minutes 200 volts using a Mini-Protean 3 cell system (Biorad) in 1X Glycine Tris running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). A 2 x 3.5 inch piece of PVDF membrane (Thermo Scientific) with dimension was soaked in methanol (EMD Chemicals, U.S.A) for 30 seconds and equilibrated in transfer buffer (25mM Tris, 192 mM Glycine, 0.1% SDS) with the gel for 30 minutes. Proteins were transferred to a PVDF membrane using a semi-dry transfer cell (BioRad) at 15 volts for 25 minutes. The PVDF membrane was

blocked overnight in 100mL 5% (w/v) non-fat dry milk in 1X PBST (0.1% [v/v] Tween-20 solution in 1X PBS) with constant slow shaking at 4°C. The PVDF membrane was incubated with either a polyclonal anti-MbaA antibody at a 1:1000 concentration in 10 ml 1X PBST for 1 hour with constant rotation, washed by three consecutive 1X PBST washes for 10 minutes each, incubated with an HRP conjugated goat anti-rabbit antibody at a 1:10,000 concentration in 10 ml 1X PBST for 1 hour with room temperature rotating and washed by three consecutive 1X PBST washes for 10 minutes each, or an anti-V5 antibody (Serotec) at a 1:10,000 concentration in 10 ml 1X PBST for 1 hour with constant rotation, and washed by three consecutive 1X PBST washes for 10 minutes each. Following the wash, the membrane was incubated with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) for 10 minutes. Membranes were exposed to X-ray film for 10, 30, and 45 seconds, respectively. Film was developed in a Konica Minolta SRX-101A developer (Konica Minolta Medical & Graphic, Inc., China).

### ***Bioinformatics***

The SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>) was used to determine where the signal peptide of NspS was located by entering the NspS FASTA sequence in amino acid format as obtained from NCBI (<http://www.ncbi.nlm.nih.gov/>). Domain architecture of MbaA was determined using the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>) by entering the MbaA protein sequence in FASTA format as obtained from NCBI.

**Table 1. Strains and Plasmids**

Strain/Plasmid	Genotype	Reference
<b><i>E. coli</i> strains</b>		
DH5α	F <sup>-</sup> , φ80d <i>lacZ</i> ΔM15, Δ( <i>lacZYA-argF</i> )U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rK <sup>-</sup> , mK <sup>+</sup> ), <i>phoA</i> , <i>supE44</i> , λ <sup>-</sup> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Invitrogen
AK261	DH5α (pET26)	This Study
AK272	DH5α (pET28b:: <i>MbaA</i> )	This Study
AK274	BL21(DE3) (pET28b:: <i>MbaA</i> )	This Study
AK276	BL21(DE3) (pET26b:: <i>NspS</i> )	This Study
<b><i>V. cholerae</i> O139 strains</b>		
PW444	MO10 Δ <i>mbaA</i> , <i>lacZ</i> :: <i>vpsLp</i> → <i>lacZ</i> , Sm <sup>r</sup>	(Karatan <i>et al.</i> , 2005)
PW514	MO10 Δ <i>nspS</i> , <i>lacZ</i> :: <i>vpsLp</i> → <i>lacZ</i> , Sm <sup>r</sup>	(Karatan <i>et al.</i> , 2005)
PW522	MO10 Δ <i>nspS</i> , Δ <i>mbaA</i> , <i>lacZ</i> :: <i>vpsLp</i> → <i>lacZ</i> , Sm <sup>r</sup>	(Karatan <i>et al.</i> , 2005)
AK007	PW514 (pACYC184)	Zayner, 2008
AK192	PW514 (pACYC184 <i>nspS</i> -V5)	This Study
AK193	PW522 (pACYC184 <i>nspS</i> -V5)	This Study
<b>Plasmids</b>		
pACYC184	Tet <sup>r</sup>	New England Biolabs
pCR2.1-TOPO	Plasmid for TOPO cloning, Amp <sup>r</sup>	Invitrogen
pNP1	pACYC184:: <i>nspS</i> v5	Zayner, 2008
pET28(b)	Plasmid vector for cytoplasmic expression of recombinant proteins	Novagen
pET26(b)	Plasmid vector for periplasmic localization and expression of recombinant proteins	Novagen
pET28b:: <i>mbaA6His</i>	pET28b:: <i>mbaA6His</i>	This study
pET26b:: <i>nspS6His</i>	pET26b:: <i>nspS6His</i>	This study

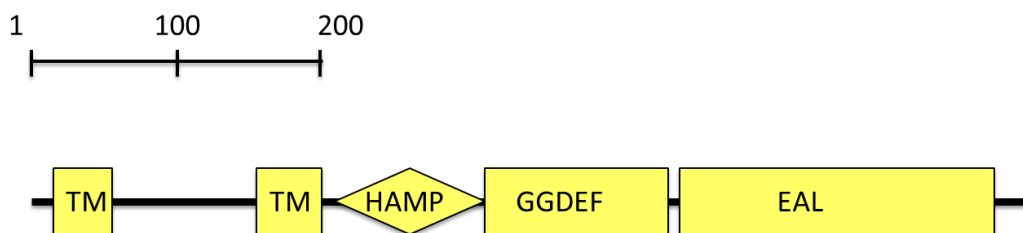
**Table 2. Primers**

Primer	Description	Sequence
P215	Forward primer 99bp upstream of <i>NspS</i>	5'-GCGACCAAATTATACATAGAG-3'
PA24	Reverse primer for <i>nspS</i>	5'-GCTGCCATGGCTACGTAGAATCG-3'
PA184	Reverse primer for pET28b:: <i>NspS</i>	5'-TGTCTCGAGTGGTTTAGCTTCATATTGG-3'
PA195	Forward primer for pET28b:: <i>MbaA</i>	5'-AAGCATATGGTGATCAATCCGATCCTGC-3'
PA196	Reverse primer for pET28b:: <i>MbaA</i>	5'-TGTCTCGAGCTAACGGCATTCACTTTGGC-3'
PA197	Forward primer for pET26b:: <i>NspS</i>	5'-AAGCCATGGTGGAAGTCAATCTCTACCTT-3'

## RESULTS

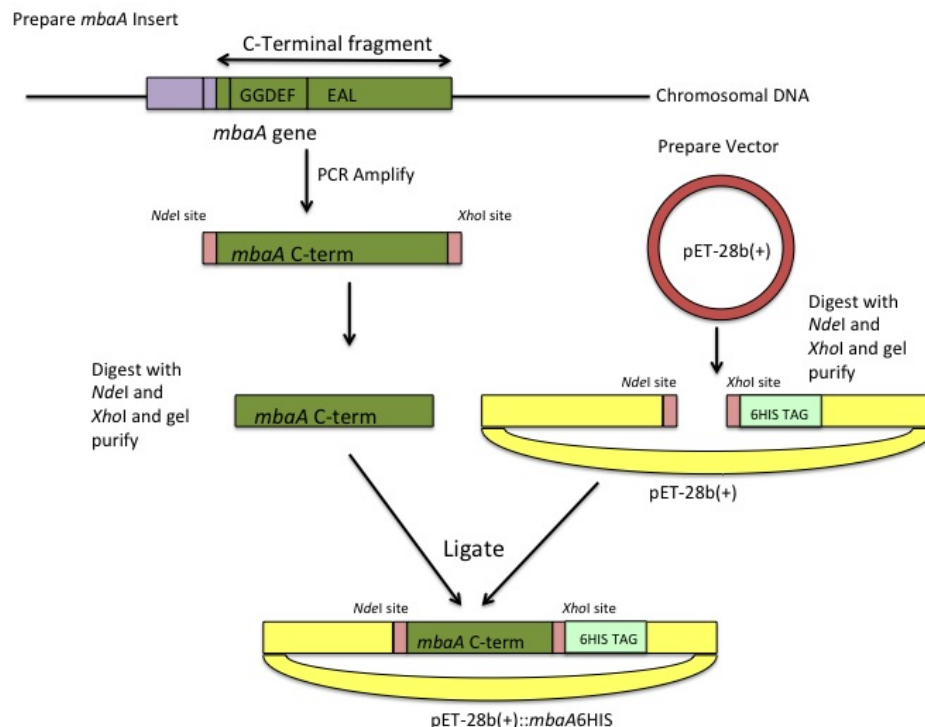
### *Construction of plasmid for expression of the c-terminal fragment of MbaA*

In an effort to provide conclusive evidence of an interaction between NspS and MbaA, I determined that coimmunoprecipitation experiments would allow me to show interaction between the two proteins in a straightforward manner. Previous work in the lab resulted in the engineering of a V5 epitope tag onto the NspS protein for which a commercial antibody was readily available (Zayner, 2008). The primary complication of this experiment was that we did not have an available antibody for the MbaA protein. To solve this problem, the pET-28b(+) cytoplasmic expression system was utilized for the cloning, expression, and purification of the MbaA protein. This protein would be used directly for immunization into rabbits for polyclonal antibody production. The MbaA protein is a large integral membrane protein with multiple domain motifs (Figure 2A). Specifically, the domain architecture of the MbaA protein includes GGDEF and EAL domains known to function in c-di-GMP signaling, a HAMP linker domain (present in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins, and Phosphatases) often found in conjunction with GGDEF/EAL domains, and several transmembrane domains. It has been suggested that HAMP domains play a role in regulating the phosphorylation or methylation of homodimeric receptors by transmitting the conformational changes in periplasmic ligand-binding domains to signal kinase and methyl-acceptor domains.

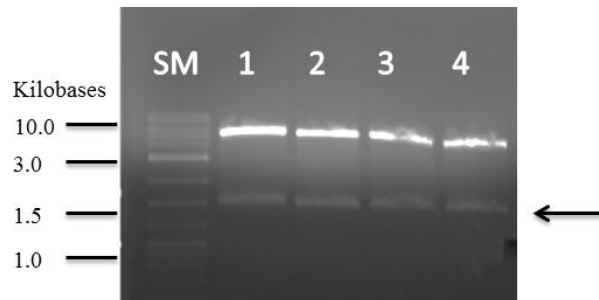


**Figure 2A. Genomic architecture and predicted cellular locations of NspS and MbaA.**

It was determined that the protein's large size and transmembrane region would make it difficult to purify in large enough amounts for use in antibody production. For this reason, the region encoding the C-terminal cytoplasmic portion of the protein containing the GGDEF and EAL domains was cloned into pET-28b(+) as described in Materials and Methods. The overall procedure involved from plasmid construction to protein purification is illustrated in Figure 2B. I cloned and ligated the MbaA C-terminal fragment. This construct was transformed into *E. coli* DH5 $\alpha$  cells as a non-expression host to propagate the plasmid and positive clones were identified using colony PCR. The plasmid was then transformed into BL21(DE3) cells. These cells carry the T7 RNA polymerase gene required for expression of the target gene using IPTG induction. Four colonies were checked for the presence of the 1500bp C-terminal fragment by restriction digest (Figure 2C).



**Figure 2B. Flowchart of cloning of a fragment encoding C-terminal MbaA protein.**



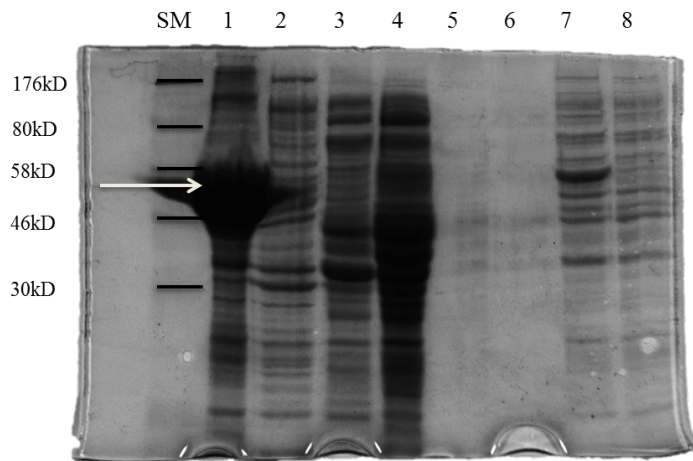
**Figure 2C. Confirmation of the *mbaA* insert in pET-28b(+):MbaA6His construct.** Four isolated colonies were digested with *NdeI* and *XhoI* and run on a 1% agarose gel. An arrow indicates the *mbaA* insert predicted to be 1500bp. SM: size marker, lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4.

### ***Purification of MbaA C-terminal fragment for polyclonal antibody production***

Once the pET-28b(+):MbaA6His plasmid was transformed into BL21(DE3) *E. coli* cells the new strain was tested for its ability to express the recombinant protein using IPTG induction. This pilot expression was performed as outlined in the methods section.

The addition of IPTG induces transcription of the T7 RNA polymerase present in BL21(DE3) cells. This bacteriophage T7 RNA polymerase hijacks the cells' resources and devotes a significant amount of materials toward target gene expression. To facilitate verification of protein expression, cells were harvested and fractionated into total cellular protein, soluble cytoplasmic protein, insoluble cytoplasmic protein, and medium fractions. The culture medium was also checked for target protein leakage from cells since the culture was induced for an extended period of time. Uninduced controls were utilized to determine if induction and expression was successful (Figure 3).

SDS-PAGE analysis and Coomassie staining revealed that IPTG induction was successful as judged by the 55 kD band seen in the induced total cell protein fraction and not seen in the uninduced control. However, staining also determined that the bulk of the recombinant protein (>80%) was located in the insoluble cytoplasmic fraction. A limited amount of recombinant protein was seen in the soluble cytoplasmic fraction. No appreciable amounts of protein were seen in the media fractions, indicating that target protein leakage did not occur. Following this analysis the entire procedure was scaled up to induce a 1L culture. Inclusion bodies were purified from this culture as outlined in the methods, and gel slices of the recombinant MbaA inclusion body proteins were prepared using SDS-PAGE. Gel slices corresponding to approximately 2mg of C-terminal MbaA protein was used directly for polyclonal antibody production.



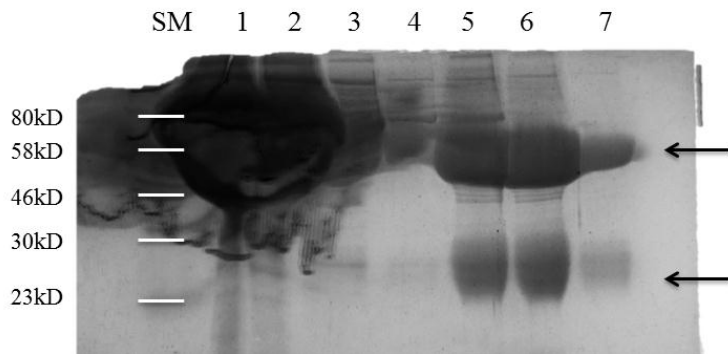
**Figure 3. Expression and localization of recombinant MbaA C-terminal protein.** Cellular fractionations were separated by SDS-PAGE and stained with Coomassie brilliant blue. An arrow indicates the MbaA protein fragment predicted to be 55kD. SM: size marker, lane 1: induced insoluble fraction (inclusion bodies); lane 2: uninduced insoluble fraction; lane 3: uninduced soluble cytoplasmic fraction; lane 4: induced soluble cytoplasmic fraction; lane 5: induced cellular media fraction; lane 6: uninduced cellular media fraction; lane 7: induced total cellular protein; lane 8: uninduced total cellular protein.

#### ***Purification of polyclonal antibodies against MbaA C-terminal protein***

In order to reduce nonspecific binding of total serum proteins during immunoblotting, immunoaffinity chromatography was used to isolate polyclonal antibodies from sera of rabbits immunized with MbaA C-terminal protein. Affinity purified antibody is necessary for accurate coimmunoprecipitation assays as nonspecific binding of contaminating serum proteins makes the isolation and detection of relevant immunoprecipitated protein complexes significantly more challenging. A Thermo Scientific Nab Spin Kit was used for affinity purification and involved the use of microcentrifuge spin columns pre-filled with immobilized protein A/G resin. Protein A/G is useful for binding a broad range of IgG subclasses as it is a recombinant fusion protein



that incorporates the IgG binding domains of both protein A and protein G. A spin purification protocol was followed as described in Materials and Methods. Non-IgG contaminants were washed from the spin column with binding buffer and the purified IgG was eluted using a pH 2.8 elution buffer. Three elution fractions were obtained and purity was determined by SDS-PAGE. The concentration of IgG in each elution fraction was determined using the IgG reference option on a NanoDrop 1000 spectrophotometer. IgG is composed of a four chain structure including two light chains at 23kD and two heavy chains at 50-70kD. Purified IgG fractions show clear presence of both heavy and light IgG chains and no other proteins, suggesting that the antibodies were highly purified from total serum proteins (Figure 4).

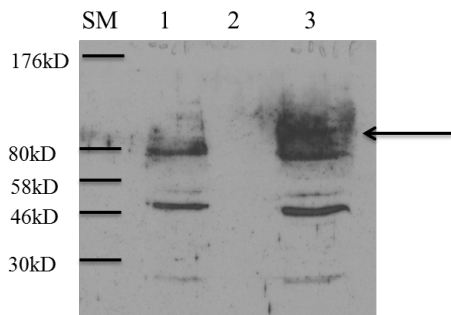


**Figure 4. Affinity purification of the polyclonal MbaA antibody.** Purification was accomplished as described in Materials and Methods. SM: size marker, lane 1: total serum; lane 2: unbound serum flow through; lane 3: wash 1; lane 4: wash 3; lane 5: elution 1; lane 6: elution 2; lane 7: elution 3. Arrows represent both IgG heavy (top) and light (bottom) chains, respectively.

#### ***Testing of anti-MbaA polyclonal antibody***

It was important to evaluate the specificity of the MbaA antibody in order to properly optimize conditions for its use both in western blotting and immunoprecipitation experiments. An Elisa was performed using purified MbaA C-terminal protein, which

showed that the antibody was specific to the purified protein. A western blot of purified protein further confirmed that the antibody was protein specific (Data not shown). We proceeded to run a western blot with the anti-MbaA affinity-purified antibody using *V. cholerae* w/ pACYC184 *nspS*-V5 and *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5 strains. The MbaA protein has a calculated mass of 91kD and the western blot revealed a band at around this size in the *V. cholerae* strain with MbaA present and a band was not seen in the strain with MbaA deleted (Figure 5.).



**Figure 5. Detection of MbaA in whole cell extracts.** The 91 kD MbaA monomer is indicated by an arrow. SM: size marker, lane 1: *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5; lane 2: buffer lane; lane 3: *V. cholerae* w/ pACYC184 *nspS*-V5.

Interestingly, several other bands of lower size were present on the western blot. Specifically two bands at approximate sizes of 80 kD and 60 kD were consistently observed during extended testing of the antibody in all *V. cholerae* lysates tested. Nevertheless, this testing provided direct evidence of a band of appropriate size for the MbaA protein that was not present in strains carrying an MbaA deletion.

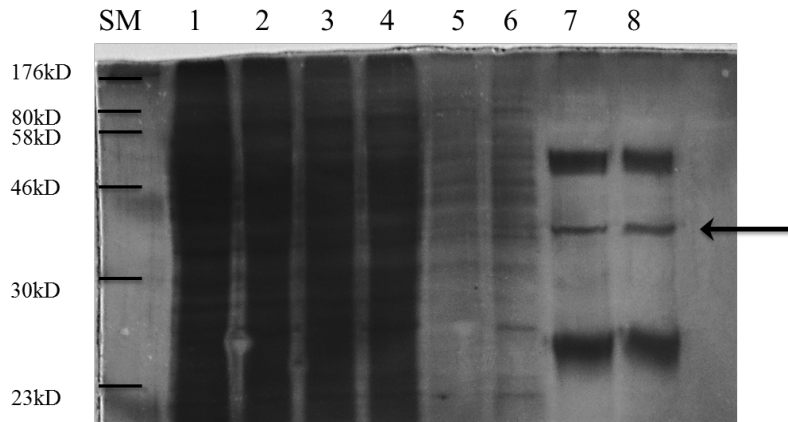
### ***Bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>) crosslinking reveals NspS dimerization and NspS/MbaA heterodimer***

Once it had been verified that the MbaA antibody was specific to purified protein, it was used to test the interaction between NspS and MbaA. Initial

coimmunoprecipitation experiments using the MbaA antibody did not reveal the presence of NspS in the MbaA immunoprecipitation complex during western blot analysis. I performed the reciprocal experiment using agarose-immobilized anti-V5 antibody in an attempt to capture the putative NspS/MbaA complex using the V5 antibody. A silver stain of this immunoprecipitation revealed that NspS was being captured by the V5 antibody, but that no other protein bands corresponding to the molecular weight of MbaA (91 kD) were observed. The two other bands present in the silver stain correspond to the heavy and light chains of the antibody IgG. (Figure 6).

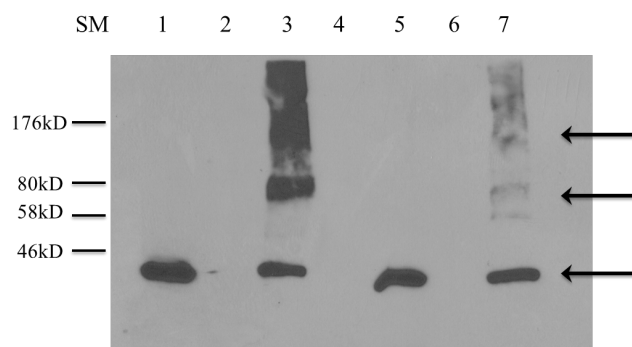
This observation led to a hypothesis that perhaps NspS and MbaA were in fact interacting but lysis and wash conditions during Co-IP were disrupting the complex and preventing our ability to pick it up with the V5 antibody. In order to overcome this potential obstacle I utilized the bifunctional N-hydroxysulfocuccinimide (NHS) ester, bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>). This crosslinking reagent, which reacts with free primary amines in proteins, is water soluble and membrane-impermeable and can effectively covalently crosslink interacting proteins on the inner membrane and the periplasmic space, hypothesized to be the location of NspS/MbaA interaction. This crosslinking reagent was incubated with both *V. cholerae* w/ pACYC184 *nspS*-V5 and *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5 strains as described in Materials and Methods. Initial crosslinking experiments were performed, followed by SDS-PAGE and Western blot with the V5 antibody. Previous use of this antibody using strains carrying the pACYC184 *nspS*-V5 plasmid consistently resulted in a single 40 kD band corresponding to a NspS monomer (Figure 6). However, upon crosslinking the same strain revealed two additional bands, one at 80 kD and another at 130 kD. We hypothesized the 80 kD band

to correspond to an NspS homodimer. Interestingly the 130 kD band was not seen in strains carrying an MbaA knockout. This led to the hypothesis that the 130 kD band corresponds to an MbaA/NspS heterodimer composed of the 91 kD MbaA protein and the 40 kD NspS protein (Figure 7.).

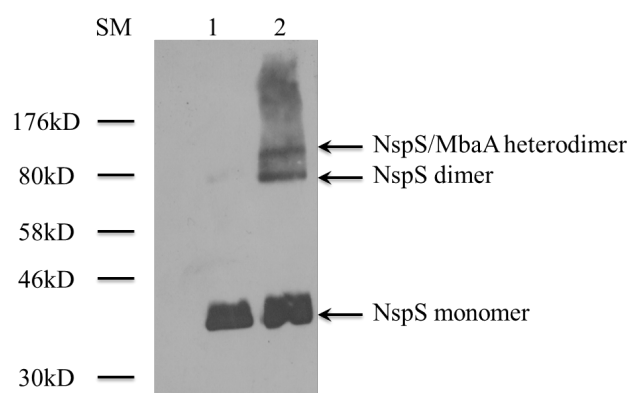


**Figure 6. Silver stain of the immunoprecipitation complex using V5 agarose.** The 40 kD NspS monomer is indicated by an arrow. SM: size marker; lane 1: Total cell protein of *V. cholerae* w/ pACYC184 *nspS*-V5; lane 2: Total cell protein of *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5; lane 3: unbound flowthrough of *V. cholerae* w/ pACYC184 *nspS*-V5; lane 4: unbound flowthrough of *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5; lane 5: wash 1 of *V. cholerae* w/ pACYC184 *nspS*-V5; lane 6: wash 1 of *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5; lane 7: IP of *V. cholerae* w/ pACYC184 *nspS*-V5; lane 8: IP of *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5.

It is important to note that protein concentrations for western blotting were always normalized and that crosslinked *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5 consistently revealed a much weaker signal for the 80 kD band even as the 40 kD band maintained an equal level of expression with the *V. cholerae* w/ pACYC184 *nspS*-V5 strain (Figure 8).



**Figure 7. Effect of BS<sup>3</sup> crosslinking on *V. cholerae* and *V. cholerae* w/ $\Delta$ *mbaA*.** SM: size marker; Lane 1: *V. cholerae* w/ pACYC184 *nspS*-V5; lane 2: buffer lane; lane 3: *V. cholerae* w/ pACYC184 *nspS*-V5 crosslinked; lane 4: buffer lane; lane 5: *V. cholerae* w/  $\Delta$ *mbaA*/pACYC184 *nspS*-V5; lane 6: buffer lane, lane 7: *V. cholerae* w/  $\Delta$ *mbaA*/pACYC184 *nspS*-V5 crosslinked. Arrows indicate the NspS monomer, homodimer, and NspS/MbaA heterodimer.

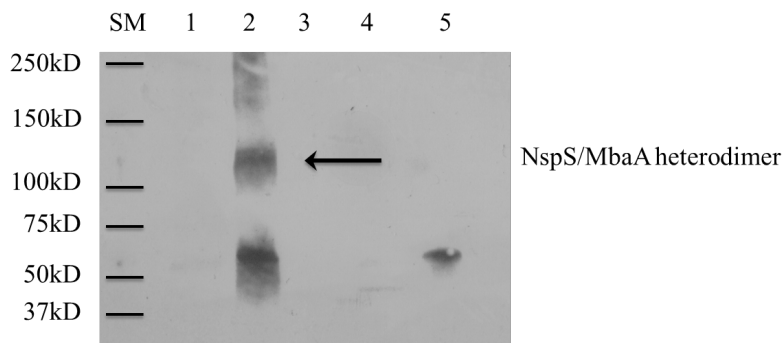


**Figure 8. Effect of BS<sup>3</sup> crosslinking on NspS dimerization and complex formation.** SM: size marker; Lane 1: crosslinked *V. cholerae* w  $\Delta$ *mbaA*/ pACYC184 *nspS*-V5; lane 2: crosslinked *V. cholerae* w/ pACYC184 *nspS*-V5.

### ***Immunoprecipitation provides evidence of NspS/MbaA interaction***

The above experiments revealed chemical crosslinking using BS<sup>3</sup> was effective in stabilizing the MbaA/NspS protein interaction in order to allow for successful co-immunoprecipitation experiments. Due to the consistent specificity of the anti-V5 antibody in revealing the NspS dimer and putative NspS/MbaA heterodimer (Figure 8) we decided to use agarose-immobilized anti-V5 antibody to capture the NspS/MbaA

complex and test for the presence of MbaA using standard western blotting procedures. The western blot revealed the presence of a single 130 kD band in the *V. cholerae* w/ pACYC184 *nspS*-V5 strain and no such band in the *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5 strain (Figure 9). It is especially compelling that the same 130 kD band was picked by the anti-V5 antibody upon on a subsequent western blot using remaining cell sample from the previous western blot.



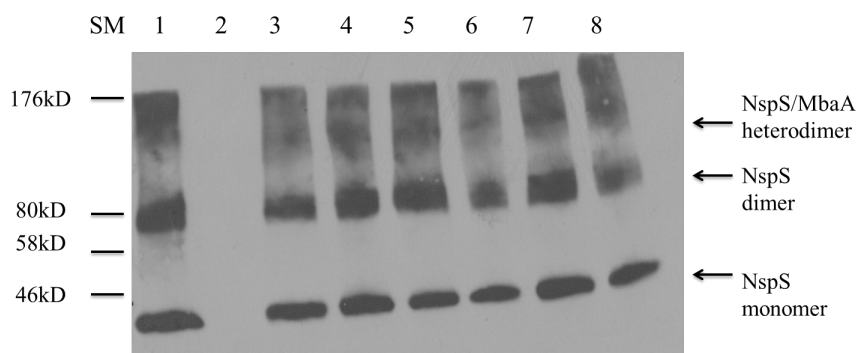
**Figure 9. Co-immunoprecipitation of NspS/MbaA using anti-MbaA antibody.** The 130 kD MbaA heterodimer is indicated by an arrow. SM: size marker; Lane 1: IP flowthrough of *V. cholerae* w/ pACYC184 *nspS*-V5; lane 2: IP of *V. cholerae* w/ pACYC184 *nspS*-V5; lane 3: buffer lane; lane 4: IP flowthrough of *V. cholerae* w/  $\Delta mbaA$ / pACYC184 *nspS*-V5; lane 5: IP of *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5.

This experiment clearly demonstrated that the covalent crosslinking of proteins in both *V. cholerae* strains was effectual in maintaining the NspS/MbaA complex during wash/lysis steps that were potentially disrupting the complex without the presence of the crosslinker. This phenomenon reveals the MbaA/NspS association to be potentially weak and possibly transient. It was difficult to determine from the western blot but a light smear above the 130 kD band may further correspond to a possible heterotetramer of MbaA/NspS; however, this possibility has yet to be confirmed experimentally. Attempts to stain an SDS-PAGE gel of these crosslinked co-immunoprecipitations with Coomassie

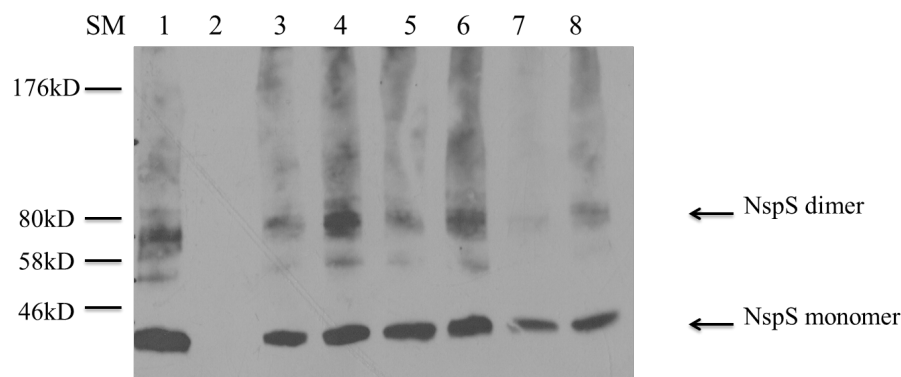
Brilliant Blue have been inconclusive due to extremely faint bands in the gel. If we could pick up bands at relevant sizes using SDS-PAGE we could potentially use MALDI-TOF mass spectrometry to determine exactly what proteins are present in the sample.

***Addition of polyamines may cause a conformational change in the MbaA/NspS complex but does not affect complex formation***

Previous studies have revealed that exogenous norspermidine increased biofilm formation in a NspS-dependent manner and that norspermidine was a signal under these conditions (Karatan *et al.*, 2005). Further spermidine addition significantly inhibits biofilm formation in an MbaA-dependent manner (McGinnis, 2009). Since MbaA and NspS are believed to interact in order to affect biofilm formation I hypothesized that performing co-immunoprecipitation experiments using *V. cholerae* w/ pACYC184 *nspS*-V5 and *V. cholerae* w/  $\Delta mbaA$ /pACYC184 *nspS*-V5 grown as overnight cultures with the presence of increasing norspermidine or spermidine concentrations may provide insight into this effect. Immunoprecipitations were performed as described in Materials and Methods and the western blots were probed with anti-V5 antibody to determine if an appreciable effect on dimerization and complex formation could be determined. The results of this experiment suggested that there was no perceptible change in the effect of dimerization and complex formation resulting from increasing polyamine concentrations (Figure 10A, 10B). However, as stated previously the amount of dimerization and other complex formation was significantly less in the MbaA knockout strain.



**Figure 10A. Polyamine effect on MbaA/NspS complex in *V. cholerae*.** SM: size marker, lane 1: *V. cholerae* w/ pACYC184 *nspS*-V5; lane 2: buffer lane; lane 3: 100  $\mu$ M spermidine; lane 4: 500  $\mu$ M spermidine; lane 5: 1000  $\mu$ M spermidine; lane 6: 100  $\mu$ M norspermidine; lane 7: 500  $\mu$ M norspermidine; lane 8: 1000  $\mu$ M norspermidine.

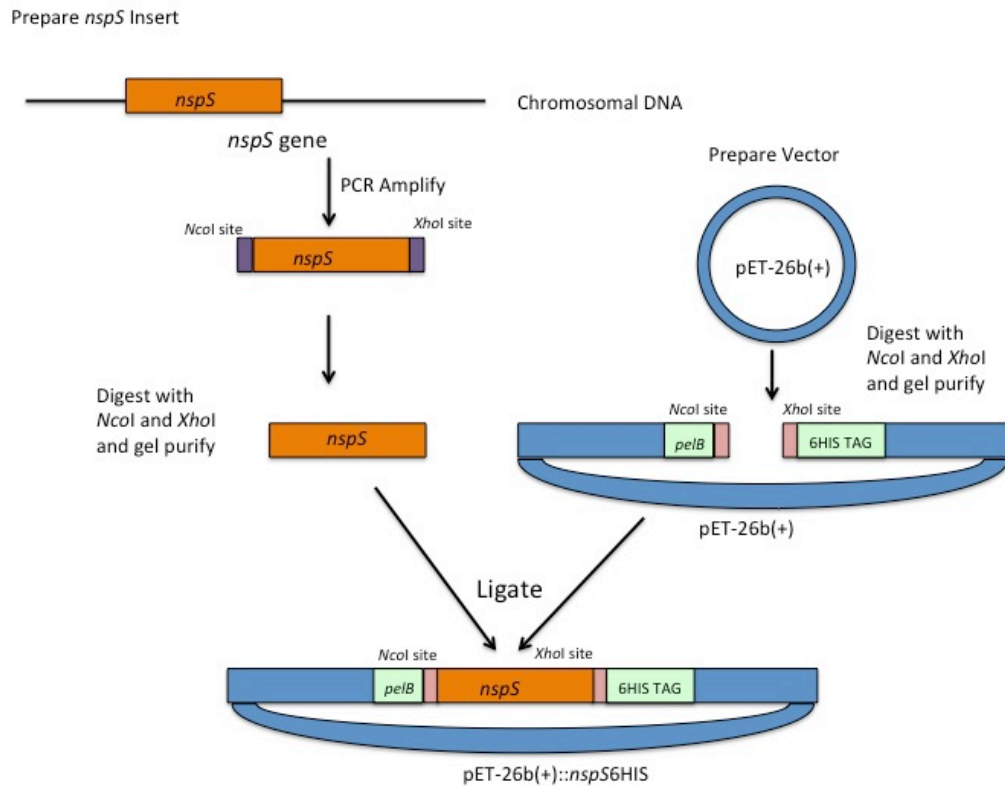


**Figure 10B. Polyamine effect on MbaA/NspS complex in *V. cholerae* w  $\Delta$ *mbaA*.** SM: size marker, lane 1: *V. cholerae* w  $\Delta$ *mbaA*/ pACYC184 *nspS*-V5; lane 2: buffer lane; lane 3: 100  $\mu$ M spermidine; lane 4: 500  $\mu$ M spermidine; lane 5: 1000  $\mu$ M spermidine; lane 6: 100  $\mu$ M norspermidine; lane 7: 500  $\mu$ M norspermidine; lane 8: 1000  $\mu$ M norspermidine.

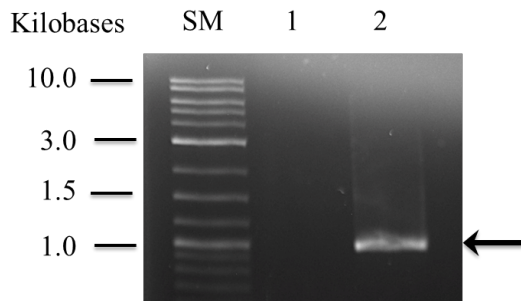
### ***Construction of plasmid for expression of NspS***

In an effort to determine whether norspermidine and spermidine are ligands to NspS a pET-26b(+) periplasmic expression system was utilized for the cloning, expression, and purification of NspS protein for use in ligand binding assays (Figure 11).





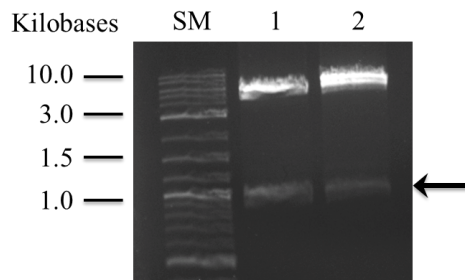
**Figure 11. Flowchart of cloning and ligation of the *nspS* gene using pET-26b(+).**



**Figure 12. Amplification of *nspS* insert using Phusion® Polymerase.** The 1080 bp insert is indicated by an arrow. SM: size marker; lane 1: buffer lane; lane 2: *nspS* insert.

The 1080bp *nspS* gene was PCR-amplified from chromosomal *V. cholerae* DNA (Figure 12). This insert was purified and cloned into pET-26b(+) as described in Materials and Methods. The overall procedure involved from plasmid construction to protein purification is illustrated in Figure 10. This construct was transformed into *E. coli*

DH5 $\alpha$  cells as a non-expression host to propagate the plasmid, and positive clones were identified using colony PCR. The plasmid was then transformed into BL21(DE3) cells for expression of the target gene using IPTG induction. DNA sequencing was used to verify that the entire insert sequence was correct with no mutations and isolated plasmids were verified by restriction digest to confirm the presence of the *nspS* insert. (Figure 13).

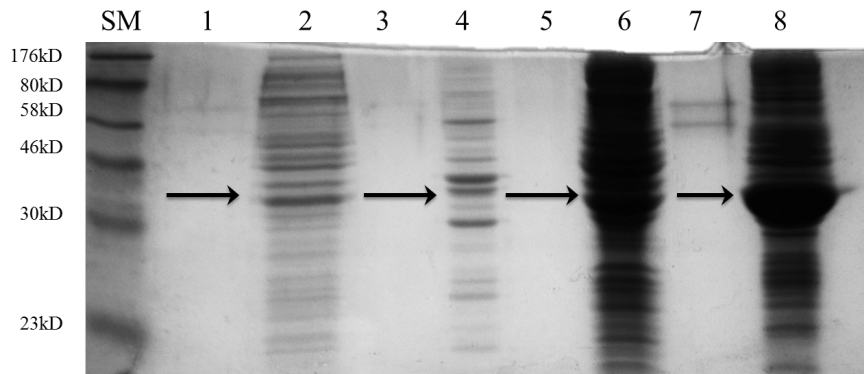


**Figure 13. Confirmation of the *nspS* insert in pET-26b(+):NspS6His construct.** Two isolated colonies were digested with *Nco*I and *Xho*I and run on a 1% agarose gel. The *nspS* insert predicted to be 1080bp is indicated by an arrow. SM: size marker, lane 1: colony 1; lane 2: colony 2.

Since NspS is a periplasmic protein we decided to use the pET-26b(+) vector since it encodes an N-terminal *pelB* signal sequence for periplasmic localization of the recombinant protein and an additional C-terminal polyhistidine tag for affinity purification. In an effort to acquire meaningful data we believed that only NspS protein that was shuttled to the periplasm and subsequently processed could be definitively expected to exist in its native folded conformation. We judged that only natively folded soluble protein would be effective for binding assays since this conformation most closely resembles what we hypothesize to be the conformation NspS exists as during its interaction with MbaA.

### ***Purification of NspS for use in ligand binding studies***

Once the pET-26b(+):NspS6His plasmid was transformed into BL21(DE3) *E. coli* cells the new strain was tested for its ability to express the recombinant protein using IPTG induction. This pilot expression was performed as outlined in the methods section. To facilitate protein verification a small-scale analysis was performed using cells harvested and fractionated into total cellular protein, soluble cytoplasmic protein, insoluble cytoplasmic protein, and periplasmic proteins. SDS-PAGE analysis and Coomassie staining revealed that IPTG induction was successful as judged by the notable 40 kD band seen in all fractions. However, staining also determined that the bulk of the recombinant protein (>80%) was located in the insoluble cytoplasmic fraction similarly to the pET-28b(+):MbaA6His construct. A significant amount of NspS was also found in the periplasmic fraction as expected for this vector (Figure 14.).

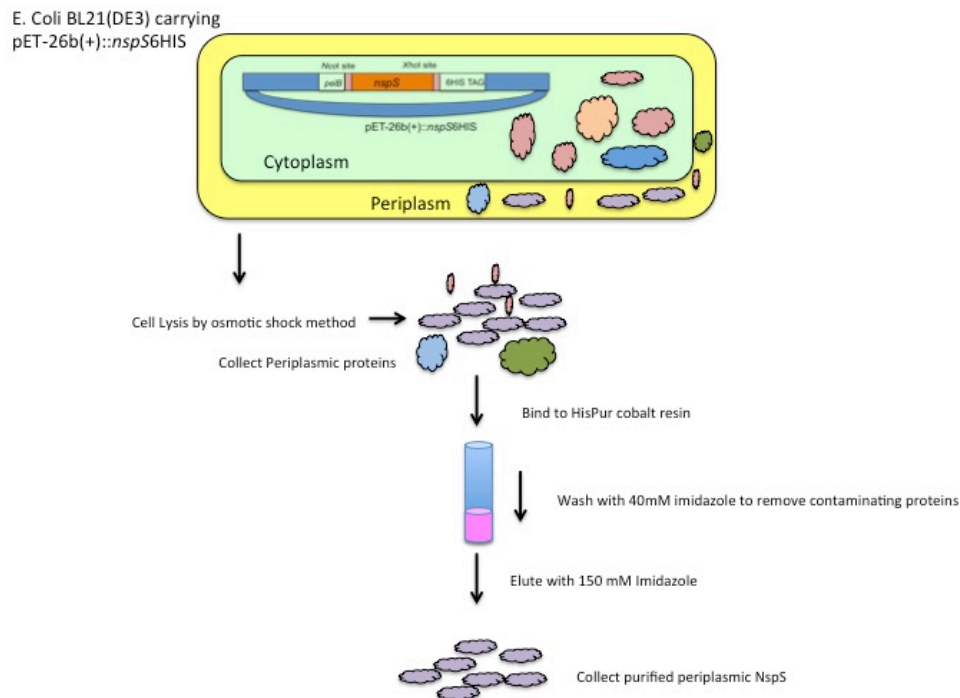


**Figure 14. Expression and localization of recombinant NspS protein.** Cellular fractionations were separated by SDS-PAGE and stained with Coomassie brilliant blue. The NspS protein predicted to be 40kD is indicated by an arrow. SM: size marker, lane 1: buffer lane; lane 2: total cellular protein fraction; lane 3: buffer lane; lane 4 induced periplasmic fraction; lane 5: buffer lane; lane 6: induced soluble cytoplasmic fraction; lane 7: buffer lane; lane 8: induced insoluble cytoplasmic fraction (inclusion bodies).

Following this analysis the entire procedure was scaled up to induce a 1L culture.

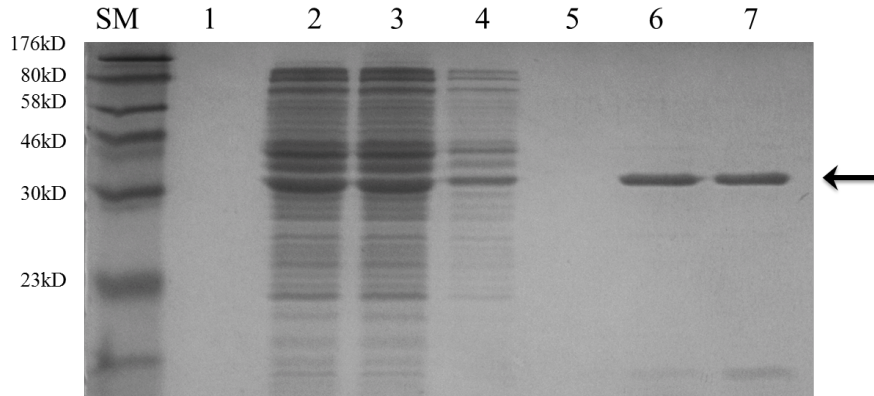
Periplasmic proteins were obtained using the osmotic shock method outlined in Materials

and Methods. These proteins were concentrated to 10 mL and dialyzed extensively against a lysis buffer containing 50mM sodium phosphate, 300mM sodium chloride, and 30mM imidazole at pH 7.4. Following dialysis NspS was purified from total periplasmic proteins using metal affinity chromatography. HisPur cobalt resin was used for this purpose. HisPur is a tetradentate chelating resin charged with divalent cobalt ( $\text{Co}^{2+}$ ) that allows for purification of histidine-tagged recombinant proteins due to the affinity of histidine for the metal ions. Cobalt resin was chosen over nickel-NTA due to cobalt's higher specificity for histidine-tagged proteins with lower nonspecific binding. A general procedure was completed following isolation and dialysis of periplasmic proteins from BL21(DE3) carrying the pET-26b(+):NspS6His plasmid as per manufacturer's instructions and is outlined in figure 15.



**Figure 15. General purification scheme using HisPur cobalt resin to purify NspS.**

The wash conditions were optimized from 5 mM imidazole to 40 mM in order to remove nonspecifically bound contaminating proteins. Following the completed purification, all fractions were analyzed using SDS-PAGE in order to evaluate protein purity (Figure 16). The single large band seen in each elution fraction confirmed the purity of the periplasmic NspS protein. The elution fractions were then dialyzed to remove high imidazole concentrations and stored for use in binding studies with its proposed ligands, norspermidine and spermidine.



**Figure 16. Purification of periplasmic NspS protein using HisPur cobalt resin.** The NspS protein predicted to be 40kD is indicated by an arrow. SM: size marker, lane 1: buffer lane; lane 2: periplasmic protein flowthrough; wash 1; lane 4: wash 3; lane 5: buffer lane; lane 6: elution 1; lane 7: elution 2.

## DISCUSSION

In this work I have further characterized NspS and MbaA, two protein constituents of a putative polyamine responsive signaling system in *V. cholerae* O139. The purpose of this study has been to explore several aspects of this putative signaling system. Specifically, I aimed to 1: provide evidence for NspS/MbaA interaction, and 2: to establish that spermidine and norspermidine are ligands for NspS. I engineered a recombinant MbaA C-terminal protein fragment and used it to generate a polyclonal antibody to MbaA. This antibody was used together with the membrane-impermeable crosslinking reagent BS<sup>3</sup> to provide direct evidence of an interaction between these two proteins. The crosslinking reagent in conjunction with a V5 epitope engineered on the NspS protein revealed that NspS forms a dimer and that this dimerization is significantly increased in the presence of MbaA. I also determined from Western blot analysis of crosslinked strains that a protein band at approximately 130 kD likely corresponds to an NspS (40 kD) and MbaA (91 kD) heterodimer since this band was not seen in strains carrying an MbaA deletion. These results further indicated that while previous studies show that exogenous norspermidine increases *V. cholerae* biofilm formation in an NspS and MbaA-dependent manner, crosslinking studies performed on cell cultures grown in the presence of increasing norspermidine and spermidine levels did not reveal appreciable differences in their dimerization profiles. This result indicated that the polyamines could be affecting NspS by causing a conformational change in the protein

that could alter its interaction with MbaA but not change its capacity to dimerize. Finally, I was able to develop a recombinant NspS protein construct using the periplasmic expression vector pET-26b(+). Previous studies indicate that NspS is necessary to moderate the effects of spermidine and norspermidine on *V. cholerae* biofilm formation. NspS is annotated as belonging to a family of periplasmic polyamine-binding proteins. This data taken together suggests a likelihood of norspermidine and spermidine acting as direct ligands for NspS. Since this hypothesis has never been proven, we hope to test this prediction using these polyamines and purified NspS protein with isothermal titration calorimetry.

In *V. cholerae*, the switch between biofilm and planktonic lifestyles is a tightly regulated process. A complex system of signaling pathways is used to mediate this transition, and uses a wide range of environmental cues as inputs. The basis and central regulator of this regulatory system is the bacterial second messenger, cyclic-di-GMP. Specifically, GGDEF and EAL domain proteins direct intracellular c-di-GMP levels in response to environmental signals, and c-di-GMP levels control the transition between biofilm and planktonic lifestyles.

The *V. cholerae* genome encodes a total of 61 proteins directly involved in c-di-GMP synthesis and degradation. These include 12 with EAL domains, 31 with GGDEF domains, 10 with tandem GGDEF/EAL domains, and 9 with HD-GYP domains (Heidelberg *et al.*, 2000). Due to the vast amount of these domains present in *V. cholerae*, it is easy to assume that a plethora of environmental signaling inputs should be available for initiating cyclic-di-GMP regulatory networks. However, to date only a handful of environmental signals believed to be transmitted and perceived by c-di-GMP signaling

systems have been elucidated.

Within many of the currently studied c-di-GMP biofilm regulatory systems the central c-di-GMP-dependant regulator is a specific phosphodiesterase enzyme. Previous work in our lab has demonstrated that increased levels of the polyamine norspermidine lead to increases in biofilm formation in *V. cholerae* through the action of the periplasmic protein NspS (Karatan *et al.*, 2005). The *V. cholerae* transmembrane protein MbaA is part of a group of GGDEF and EAL domain-containing regulatory proteins. MbaA is believed to function in the cyclic-di-GMP biofilm regulatory pathway since an absence of the *mbaA* gene leads to an increase in both biofilm proportion and transcription of genes encoding proteins utilized in *Vibrio* polysaccharide synthesis (Bomchil *et al.*, 2003; Karatan *et al.*, 2005). These *vps* genes are fundamental biofilm elements functioning in the biofilm regulatory pathway. The NspS protein is also proposed to function in this pathway since its ability to sense the polyamine norspermidine is associated with biofilm regulation, and its deletion leads to a drastic decrease in biofilm. MbaA is a known repressor of biofilm formation and *vps* gene expression and so it is likely that MbaA functions as a phosphodiesterase to break down c-di-GMP (Bomchil *et al.*, 2003; Karatan *et al.*, 2005; Karatan & Watnick, 2009). Since NspS is known to promote biofilm formation and MbaA is known to hinder biofilm formation, it is likely that inhibition of MbaA activity is mediated by NspS via a putative NspS/MbaA signaling system reactive to polyamines that works to regulate biofilm formation, *vps* gene expression, and cyclic-di-GMP levels in *V. cholerae*.

In order to address the first specific aim of this study, we utilized co-immunoprecipitation experiments. Co-immunoprecipitation is a proven method to



address protein-protein interactions that uses an antibody to immunoprecipitate a specific known protein antigen and co-immunoprecipitate any interacting proteins that may be part of larger protein signaling complexes. Initial co-immunoprecipitation assays did not indicate that NspS and MbaA were interacting due to the inability to pick up an NspS/MbaA complex using standard co-immunoprecipitation protocols. In order to overcome this initial setback, I decided to crosslink the cell lysates using the NHS ester BS<sup>3</sup>. I discovered that upon crosslinking the *V. cholerae* strain containing the V5-tagged NspS protein and probing for the V5-tagged NspS using immunoblotting, several bands emerged that had not been seen previously. Without crosslinking I saw one band at 40 kD corresponding to a monomer of NspS but crosslinking revealed bands at 40 kD, 80 kD, and 130 kD. I have hypothesized the 80 kD band to be a likely NspS homodimer. The 130 kD band was not present in crosslinked cell lysates that had *mbaA* gene deletions. Since MbaA is believed to be 91 kD in size, I hypothesized that the 130 kD band corresponded to an MbaA/NspS heterodimer. In order to address this I am attempting to Coomassie stain these SDS-PAGE gels in order to use MALDI-TOF mass spectrometry to confirm my hypothesis. Presently stained protein bands are too weak to be identified by the mass spectrometer; however, attempts to concentrate the protein complexes using anti-V5 antibody immobilized agarose are currently underway. The inability to pick up the NspS/MbaA interaction under the conditions tested without the use of a chemical crosslinker indicated that the interaction is likely a transient one. Transient protein complexes are important in the regulation of signaling pathways in the cell because they enable the cell to quickly respond to external stimuli. The presence of both monomers and homodimers of NspS under crosslinking conditions indicates that both forms of the

protein may be involved in the interaction with MbaA. For example, an NspS monomer may be active during the binding process with its polyamine ligand, but an NspS dimer may be required for its interaction with MbaA. It was interesting to see that when protein concentrations were normalized between samples, strains carrying an MbaA knockout consistently showed a much lower degree of NspS dimerization. Previous research in our lab has determined from cellular fractionation assays that increasing concentrations of exogenous norspermidine led to a greater amount of NspS protein partitioning into the membrane fraction from the periplasm where it is hypothesized to interact with MbaA (Zayner, 2008). Also when MbaA is deleted, NspS is not found in the membrane fraction. It would be interesting to use the crosslinker to determine which fraction the NspS homodimer is located in and if it is located in the membrane fraction whether or not exogenous norspermidine increases its presence in the membrane fraction.

NspS crosslinking experiments performed in the presence of increasing levels of spermidine and norspermidine indicated that the presence of the polyamine does not appear to drastically alter the amount of the NspS homodimer. However, since the experiment was carried out using whole cell extracts it is unknown if the amount of homodimer is altered in specific cellular locations. Again, cellular fractionations using the crosslinker could shed light on this possibility. Previous research regarding this pathway has shown that NspS and MbaA interaction is independent of norspermidine and spermidine since norspermidine is not found in LB culture media but that these polyamines likely modulate the NspS/MbaA interaction.

In order to address the second aim of this study, I decided to clone, express, and purify recombinant NspS protein for use in binding studies with its proposed polyamine

ligands: norspermidine and spermidine. This was accomplished using the Novagen pET-26b(+) vector system. The NspS protein is believed to interact directly with the polyamine norspermidine to induce a conformation change in the protein allowing interaction with MbaA. I cloned the NspS protein lacking the native signal sequence into the pET26b+ (Novagen) vector encoding a N-terminal *pelB* leader sequence for periplasmic localization of the recombinant protein in addition to a c-terminal histidine tag for affinity purification. The objective of this construct was to purify this recombinant protein and use it directly for isothermal titration calorimetry assays to determine binding affinity. If successful this experiment will establish the existence of the first polyamine-binding protein involved in signal transduction rather than transport discovered in *bacteria*, opening the door for research into an entirely novel class of proteins.

The NspS protein has a number of homologs present in a wide range of bacteria, all of which are periplasmic ATP binding cassette (ABC)-type transporter ligand-binding proteins. All of these proteins identified to date are known to either be parts of transport systems or parts of transport systems that also have a role in signaling. Two NspS homologs exist in *V. cholerae* and are designated PotD1 and PotD2. These homologs function in an ABC-type transport system used in the import of polyamines into the cell. However, the NspS protein is different. Recent evidence indicates that NspS cannot transport spermidine or norspermidine and thus is likely involved exclusively in signaling (Rutkovsky, unpublished results). This hypothesis is supported by the *nspS* gene being adjacent to *mbaA* on the chromosome (Porter, 2009). This suggests that NspS may be part of a subset of the ABC-type polyamine-binding transport protein family that is involved in signaling but not transduction. A comparative genomic analysis revealed that

*nspS/mbaA*-like gene pairs are present in the genomes of several closely related *Vibrio* species (*V. vulnificus*, *V. parahaemolyticus*) and in several distantly related proteobacteria including *Pseudomonas stutzeri*, *Hahella chejuensis*, and *Psychromonas ingrahamii* (Porter, 2009). None of these systems have been characterized to date, but the existence of these conserved gene pairs in other bacteria indicate that polyamine-responsive signaling systems that use c-di-GMP may be widely utilized by bacteria. Therefore, characterization of the NspS/MbaA signaling system in *V. cholerae* will provide insight into how these systems may be functioning within other bacterial species.

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## BIOGRAPHICAL SKETCH

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